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Date 16/4/87
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ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator DR. FIRDAUSI QADRI

Trainee Investigator (if any) —

Application No. 87-010 (Revised)

Supporting Agency (if Non-ICDDR,B) USAID

Title of Study : Comparison of OMP associated antigens from virulent and avirulent strains of Shigella dysenteriae type 1 and Shigella flexneri 2a

Project status:
(x) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

Circle the appropriate answer to each of the following (If Not Applicable write NA).

1. Source of Population:
   (a) Ill subjects — Yes (No)
   (b) Non-ill subjects — Yes (No)
   (c) Minors or persons under guardianship — Yes (No)
2. Does the study involve:
   (a) Physical risks to the subjects — Yes (No)
   (b) Social Risks — Yes (No)
   (c) Psychological risks to subjects — Yes (No)
   (d) Discomfort to subjects — Yes (No)
   (e) Invasion of privacy — Yes (No)
   (f) Disclosure of information damaging to subject or others — Yes (No)
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) — Yes (No)
   (b) Use of fetal tissue or abortus — Yes (No)
   (c) Use of organs or body fluids — Yes (No)
4. Are subjects clearly informed about:
   (a) Nature and purposes of study — Yes (No)
   (b) Procedures to be followed including alternatives used — Yes (No)
   (c) Physical risks — Yes (No)
   (d) Sensitive questions — Yes (No)
   (e) Benefits to be derived — Yes (No)
   (f) Right to refuse to participate or to withdraw from study — Yes (No)
   (g) Confidential handling of data — Yes (No)
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure — Yes (No)
5. Will signed consent form be required:
   (a) From subjects — Yes No NA
   (b) From parent or guardian (if subjects are minors) — Yes No NA
6. Will precautions be taken to protect anonymity of subjects — Yes No NA
7. Check documents being submitted herewith to Committee:
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   ___ Protocol (Required)
   ___ Abstract Summary (Required)
   ___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   ___ Informed consent form for subjects
   ___ Informed consent form for parent or guardian
   ___ Procedure for maintaining confidentiality
   ___ Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary
1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitive areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

No human subject is involved.

We agree to obtain approval of the Ethical Review Committee for any changes involving the rights and welfare of subjects before making such change.

Firdausi Qadri
Principal Investigator

MAY 25 1987

Trainee

SECTION I - RESEARCH PROTOCOL

87-010 (Revised)
16/4/87
19/5/87

1. TITLE : Comparison of OMP associated antigens from virulent and avirulent strains of *Shigella dysenteriae* type 1 and *Shigella flexneri* 2a

2. PRINCIPAL INVESTIGATORS : Dr. Firdausi Qadri
University of Dhaka

CONSULTANT : Dr. Ivan Ciznar
ICDDR,B

3. STARTING DATE : April, 1987

4. COMPLETION DATE : April, 1988

5. TOTAL DIRECT COST : US$37,840

6. SCIENTIFIC PROGRAM HEAD : Dr. D. A. Sack

This protocol has been approved by the Laboratory Sciences and Epidemiology Division.

Signature of the Program Head 

Date 7 May 1987

7. ABSTRACT SUMMARY

The aim of this study is to compare outer membrane proteins (OMPs) of virulent and non-virulent *Shigella* spp. (both *S. flexneri* and *S. dysenteriae* 1). Virulent strains will be defined as strains inducing keratoconjuctivitis in guinea pigs and this property will be correlated with the

ability to bind Congo red. In order to make direct comparison, we will use Congo red positive strains which are virulent with their respective non-virulent Congo red negative derivatives. Antisera prepared against bacterial OMP preparations will be used in crossed immunoelectrophoresis and Western-blot assays in homologous and hetrologous experiments. In addition, we will absorb the anti-virulent OMP sera with non-virulent OMP antigen, thereby making a sera specific for antigens associated with virulence. The absorbed and unabsorbed sera will also be used to determine if OMP antigens are common between *Shigella* and Congo red positive *E. coli* strains. Results from the study will help to define OMP antigens of *Shigella* and their relation to virulence which may assist in the design of a vaccine for shigellosis.

8. REVIEWERS

a) Ethical Review Committee : _____

b) Research Review Committee : _____

c) Director : _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective

To determine antigenicity of *Shigella* outer membrane proteins not related to virulence.

2. Background

Shigellosis is one of the most serious of all the specific diarrhoeas having the highest incidence in developing countries. Despite all the efforts to introduce effective interventions related to sanitary measures no significant reduction in mortality and morbidity has been achieved (31). Thus, it appears that vaccination could represent a potential intervention in the control of shigellosis; and therefore, the World Health Organization considers *Shigella* vaccine development program as priority one (WHO, Report 1982 and 1986). Construction of an effective *Shigella* vaccine, however, should be proceeded by identification of protective antigens and evaluation of immune responses against them, making the construction of the effective vaccine more likely.

In recent years, a certain progress has been achieved in construction of live attenuated oral vaccines against *Shigella* infection. Among the attenuated strains tested were colony mutants,

streptomycin-dependent organisms and mutant hybrids (Levine *et al.*, 1983). All these experiments have also brought new knowledge regarding the relation of cell surface to factors of pathogenicity.

Surface components of the outer membrane of dysentery producing bacteria play a crucial role in pathogenesis of the infection. Recent studies showed that large, 120 to 140 megadalton, plasmid determining synthesis of over 10 polypeptides is essential for invasiveness of *Shigella flexneri* (Hale, *et al.*, 1985). Further experiments demonstrated that four polypeptides of 78, 62, 43 and 38 kilodaltons (commonly referred to as a,b,c,d, respectively) located in the outer membrane are unique to invasive strains of *S. flexneri* (Watanabe & Nakamura, 1986). These polypeptides are antigenically cross-reactive and probably shared by different *Shigella* serotypes (Hale, *et al.*, 1985).

Another outer membrane component of *Shigella* which may be important for pathogenicity appears to be lipopolysaccharide. It is specified by chromosomal genes in *S. flexneri* (Gemski, *et al.*, 1972) by a 120 Mdal plasmid in *Shigella sonnei* (Kopecko, *et al.*, 1980) and by a 6 Mdal plasmid in *Shigella dysenteriae* type 1 (Watanabe & Timmis, 1984). Thus, it appears that OMPs which are major components determining

virulence of *Shigella flexneri* probably are not functioning equally in *Shigella dysenteriae* type 1. The data we have obtained from study of surface properties of *Shigellae* showed that ability to bind Congo Red is not associated only with presence of plasmid determining the synthesis of outer membrane proteins. However, Congo Red binding has been used as a marker of virulence for *Shigella flexneri*, *Aeromonas salmonicida*, *Pasteurella pestis* and others (Maurelli *et al*, 1984; Ishiguro *et al*, 1985; Surgalla *et al*, 1968; Payne and Finkelstein, 1977).

Obviously, an array of outer membrane proteins and lipopolysaccharide are important factors determining surface properties of *Shigella* spp.

Results of several studies indicate that there are OMPs which are species specific and OMPs which are shared among different members of one family. For instance, Hofstra and Dankert (1979) showed that OMPs of *E. coli* share several cross reacting antigens with *Salmonella*, *Klebsiella* and *Proteus* species. They, however, found some OMPs specific only for *E. coli*.

We assume that *Shigella dysenteriae* type 1 and *Shigella flexneri* may contain common OMPs similar or identical to *E. coli* and OMPs specific only for *Shigella*.

Our ongoing studies under two protocols, Protocol No. 86-008, "Expression of antigens related to OMP in *Shigella* grown in different conditions," and Protocol No. 86-013, "Local and systemic antibody response to *Shigella* OMP in patients with dysentery. Implication for vaccine development," have indicated that immune response of patients and experimental animals is characterized by production of antibodies against a wide spectrum of antigens. Apparently other antigens than those associated with the four major OMPs, a, b, c, and d, stimulate antibody production in the host. There is a question whether antibodies specific for outer membrane proteins from non-invasive strains could be protective by interacting with *Shigella* cells and blocking the mucosal invasion. The mucosal invasion has been shown to be most important for establishment of disease (Formal, *et al.*, 1972) and for immunity (Formal, *et al.*, 1965). From the point of view of live oral vaccine development, the issue of introducing proteins not responsible for invasiveness and immunogenic components not related rather than related to virulence properties to a carrier strain appears to be acceptable.

Therefore, we intend to examine immunogenicity of outer membrane proteins of virulent and non-virulent strains of *Shigella dysenteriae* type 1 and *Shigella*

flexneri 2a. We further plan to compare OMP from *Shigella* and from *E. coli* in order to determine cross reacting antigens.

3. Rationale

A major question in vaccine development programs is related to the component of pathogen that should be incorporated into the vaccine and the ones that should be deleted. Since previous experiments have shown that immunity operates effectively at the level of the invasive process, a question was raised regarding the component of the pathogen which will be most effective in the inhibition of invasiveness. If antibodies against components not associated with virulence are effective in inhibiting invasiveness of *Shigella* spp., these antigens should be tested as suitable candidates for the genetically constructed vaccine.

This study aims to elucidate experimentally immunogenicity and antigenicity of outer membrane proteins not associated with virulence of *Shigella* and to compare their cross reactivity with *E. coli* OMPs.

B. SPECIFIC AIM

a) To purify outer membrane proteins from virulent and non-virulent strains of *Shigella dysenteriae* type 1 and *Shigella flexneri* 2a and characterize them by SDS-polyacrylamide gel electrophoresis.

b) To compare antigenicity of OMPs from defined virulent and related avirulent strains using Western-blot analysis and cross immunoelectrophoresis.

c) To extract OMPs from the specified *E. coli* strains and compare their cross-reactivity with *Shigellae*. OMPs.

C. MATERIALS AND METHODS

Strains

Two *Shigella dysenteriae* type 1 strains and two *Shigella flexneri* 2a strains will be used in this study. Both, virulent and spontaneous mutants which are Pcr⁻ will be used (Table 1). The strains will be ones that we have obtained from studies on our previous protocol (Protocol No. 86-008, entitled "Expression of antigens related to OMP in *Shigella* grown in different conditions"). Each pair of virulent and non-virulent strains has been characterized biochemically and serologically in the microbiology lab at ICDDR,B. Plasmid profile analysis (Birnboim & Doly, 1979; Meyers *et al.*, 1976) and the Sereny test (Sereny, 1955) have also been carried out. The surface properties of these strains have been studied using Congo Red binding assays (Ishiguro *et al.*, 1985) and the salt aggregation test (SAT) (Rozgonyi *et al.*, 1985). Further strains, one of EIEC, ETEC, EPEC and non-pathogenic *E. coli* will be included in the study for comparison with the *Shigella* strains. These

TABLE 1

Virulent & Avirulent Pairs of Strains to be Used in the Study

*Strain	Number	PCR	SAT M $(NH_4)_2SO_4$	Sereny	Pattern (Mdal)	**Antibiotic Resistance Pattern
S. dysenteriae type 1	26406_a	+	1.5	+	140, 6, 4, 2	Resistant to A,C,T,Sxt Sensitive to Na,K,Gm
	26406_b	-	1.5	-	140, 6, 4, 2	"
	3351_a	+	1.5	+	140, 6, 4, 2	Resistant to A,C,T,Sxt Sensitive to Na,K,Gm
	3351_b	-	1.5	-	140, 6, 4, 2	"
S. flexneri 2a	611_a	+	1.5	+	140, 2.7, 2	Resistant to only T Sensitive to rest
	611_b	-	1.5	-	2.7, 2	"
	A-18_a	+	1.5	+	140, 2.7, 2	Sensitive to all
	A-18_b	-	1.5	-	2.7, 2	"
E. coli						
EIEC	4608	+	1.5	+	140	ND
EPEC	83135	+	2.5	ND	ND	ND
ETEC	045565	+	3.0	ND	ND	ND
Non-pathogenic	36000	-	3.0	-	ND	ND

*The pigmented strain is indicated with the subscript a, whereas the non-pigmented strain is referred to as b.

The paired a and b strains were obtained after inoculation of the parent strains in TSA-Congo Red plates containing 0.03% dye.

**Antibiotics used were:
A ---> Ampicillin
C ---> Chloramphenicol
T ---> Tetracycline
Sxt ---> Sulphamethoxazole and Trimethoprim
Na ---> Nalidixic acid
K ---> Kanamycin
Gm ---> Gentamycin

ND - not determined

strains at present stored at -70°C will be subcultured and tested again before use for confirmation of these properties.

Media and Culture Conditions

Bacteria will be grown in trypticase soya broth at 37°C with shaking for 12-14 hours.

OMPs

Outer membrane proteins will be extracted by a modified water extraction procedure (Oaks *et al.*, in press). Proteins will be concentrated by dialysis against polyethylene glycol and also by freeze-drying. Proteins will be reconstituted in 10mM Tris HCl pH 7.8 in the concentrations required for use in different assays and then stored in aliquots at -20°C. Proteins will be measured by the dye binding method of Bradford (1976).

Contamination of the OMPs by cytoplasmic enzymes (Kabir, 1980) perisplasmic materials (Hirst, T.R., 1986) and LPS (Osborn, 1963) will be determined after each extraction. Amount of *Shigella* toxin (Verotoxin 1) and Verotoxin 2 present in the outer membrane proteins will be detected. VT1 will be assayed using enzyme-linked immunosorbent assay (Donohue-Rolfe *et al.*, 1986) with monoclonal antibodies and pure shiga toxin, obtained by courtesy of Dr. M. Bennish and

Dr. G. Keusch; and VT1 and VT2 will be assayed using the HeLa cytotoxicity assay with neutralization using specific antibodies.

Analysis of OMPs

Outer membrane proteins will be separated by electrophoresis on 15% SDS-polyacrylamide gels (Laemmli, 1970). Electrophoresis will be carried out overnight with water cooling and at low current. Low molecular weight protein standards obtained from Pharmacia will be used as markers for estimation of subunit size. Gels will be stained for protein using Coomassie blue. Carbohydrate containing protein antigens, as well as LPS will be detected by the sensitive silver staining technique of Tsai and Frasch (1982).

Preparation of Rabbit Antisera

Adult albino rabbits will be immunized with outer membrane protein using a schedule which we have established in our laboratory. The rabbits will receive totally four or five doses of purified OMPs (total 400 µg protein) which will be administered intravenously. The first two doses (50 µg in 250 µl normal saline) will be given at an interval of 7 days. The last two doses (100 µg in 500 µl normal saline) will be given at intervals of three days. Four to five days later serum will be analyzed to determine quality of antisera. The last dose will be repeated if satisfactory

antisera is not raised. Finally, antisera will be collected and stored in aliquots at -20°C. Antisera will be raised in this manner against OMPs from all *Shigellae* and *E. coli* strains to be used in this study. At least 4 rabbits will be used for each strain. Before immunization, the serum of rabbit will be taken and tested for presence of antibodies against *Shigella* and *E. coli* OMPs.

Crossed Immunoelectrophoresis

Crossed immunoelectrophoresis of OMPs from each strain will be carried out against antisera raised in rabbits using methods described by Kroll (1973) and modified and established in our laboratories.

Western-blotting

Proteins separated on 15% SDS-PAGE will be transferred to nitrocellulose membranes under high current (360 mA) for about 1.5-2.0 hours in the Bio-Rad Trans Blot apparatus (Towbin *et al.*, 1979). The transferred protein antigen will be detected by a enzyme immunoassay technique involving alkaline phosphatase.

Densitometric Analysis

Molecular weights of protein bands on SDS-PAGE and antigens on nitrocellulose membrane will be estimated by densitometric scanning using the E.C. Densitometer (E.C. Apparatus Corporation).

Absorption Studies

Antisera will be raised in rabbits against OMPs from virulent and non-virulent *Shigella* strains. Absorption of antibodies specific for proteins from virulent strains will be done by OMP preparation obtained from non-invasive strains. It is expected that the absorbed antisera will recognize only OMPs associated with virulence, and therefore, will be suitable for identification of antigens in Western-blot analysis and crossed immunoelectrophoresis. Further, we plan to separate the protein components using the FPLC system. Both, gel filtration columns (Sephadex, Sephacryl) and ion exchange columns (Mono Q, Mono S) will be used.

D. SIGNIFICANCE

Despite the fact that genetic studies have substantially contributed to the understanding of pathogenicity of *Shigella* sp., the immunological mechanism involved in protection against *Shigella* infections are not clear. From the point of view of vaccine development, a crucial problem appears to be the identification of the component of the pathogen which stimulates protective immunity. The present study aims to identify *Shigella* OMPs which are immunogenic but, by themselves, do not confer virulence. Such peptides would be suitable candidates to be introduced into a carrier strain and utilized in a live oral vaccine against shigellosis.

It is expected that results obtained in this study will help to understand the antigenicity of *Shigella* OMPs not associated with virulence and to determine their cross reactivity with *E. coli* OMPs.

E. FACILITIES REQUIRED

No additional facility would be needed.

F. COLLABORATION

This protocol is a collaborative one between the Department of Biochemistry, University of Dhaka, and ICDDR,B. Dr. Firdausi Qadri will carry out the study in facilities available at ICDDR,B laboratories. We expect that such collaboration would continue as a base for program leading to M.S. degrees for students from University of Dhaka.

REFERENCES

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ICDDR,B
BUDGET PROPOSAL

PROGRAM NAME : LABORATORY SCIENCES & EPIDEMIOLOGY DIVISION

PROGRAM HEAD : DR. DAVID A. SACK

PROTOCOL : Comparison of OMP associated antigens from virulent and avirulent strains of *Shigella dysenteriae* type 1 and *Shigella flexneri* 2a

PRINCIPAL INVESTIGATOR: Dr. Firdausi Qadri

PROTOCOL NO.: STARTING : April, 1987

BUDGET CODE: COMPLETION : April, 1988

BUDGET SUMMARY

A/c	CATEGORY	EXPENSE 1987 0	EXPENSE 1988 0	EXPENSE 1989 0	TOTAL PROJECT COST
3100	Local Salaries	0	0	0	11340
3200	International Salaries	0	0	0	0
3300	Consultants	0	0	0	6200
3500	Travel: Local	0	0	0	0
3600	Travel: International	0	0	0	0
3700	Supplies & Materials	0	0	0	10000
3800	Other Costs	0	0	0	5100
4800	Inter-departmental	0	0	0	5200
	TOTAL DIRECT COST	0	0	0	37840
0300	Capital Expenditure	0	0	0	0
	TOTAL PROJECT COST	0	0	0	37840

PERSONNEL REQUIREMENT - LOCAL (A/c 3100)

		No. of positions	Man Months	$ Amount
(A)	Existing	0	0	0
(B)	New Recruitments	4	42	9540
(C)	Allocated from other area	1	6	1800
	SUBTOTAL	5	48	11340
(D)	Separations	0	0	0
(E)	Allocated to other area	0	0	0
	SUBTOTAL	0	0	0
	TOTAL	5	48	11340

LOCAL STAFF: (B) NEW RECRUITMENTS

Job designation	No. of position	Man month	Rate per month	$ Amount
Sr. Research Officer, GS-6	1	12	300	3600
Research Officer, GS-5	2	18	270	4860
Laboratory Attendant, GS-1	1	12	90	1080
	0	0	0	0
	0	0	0	0
TOTAL	4	42		9540

LOCAL STAFF: (C) ALLOCATED FROM OTHER AREA

Budget	Job Desig	Level	No. of position	Man month	Rate per month	$ Amount
220110	Secretary	GS-6	1	6	300	1800
			0	0	0	0
			0	0	0	0
	TOTAL		1	6		1800

CONSULTANTS (A/c 3300)

Job designation	No. of days	Total Per diem	Total Honorarium	Travel cost	$ Amount
Consultant, P.I.	365	0	6000	200	6200
	0	0	0	0	0
	TOTAL				6200

SUPPLIES AND MATERIALS (A/c 3700)

Account	Items	$ Amount
3701	Drugs	0
3702	Glassware	800
3703	Hospital Supplies	100
3704	Stationery and Office Supplies	500
3705	Chemicals and Media	1000
3706	Materials for Uniform	100
3707	Fuel, Oil and Lubricants	100
3708	Laboratory Supplies	500
3709	Housekeeping Supplies	200
3710	Janitorial Supplies	200
3711	Tools and Spares	0
3712	Non-stock Supplies	5000
	SUBTOTAL	8500
3713	Freight and Other Charges (30%)	1500
	TOTAL	10000

OTHER COSTS (A/c 3800)

Account	Items	$ Amount
3800	Repairs and Maintenance	5000
3900	Rent, Communication and Utilities	0
4100	Bank Charges	0
4200	Legal and Professional Expenses	0
4300	Printing and Publication	0
4400	Entertainment, Hospitality and Donation	100
4500	Service Charges	0
4600	Staff Development and Training	0
	TOTAL	5100

INTER-DEPARTMENTAL SERVICES (A/c 4800)

Account	Items	$ Amount
4801	Computer	100
4802	Transport - Dhaka	0
4803	Transport - Matlab	0
4804	Water Transport - Matlab	0
4805	Transport - Teknaf	0
4806	Xerox and Mimeograph	300
4807	Pathology	0
4808	Microbiology Tests	0
4809	Biochemistry	0
4810	X-ray	0
4811	I.V. Fluid	0
4812	Media	2000
4813	Patient Hospitalization - Study	0
4814	Animal - Research	2500
4815	Medical Illustration	200
4817	Telex	100
4818	Outpatient Care	0
4830	Transport Subsidy	0
	TOTAL	5200

ABSTRACT SUMMARY

The aim of this study is to compare outer membrane proteins (OMPs) of virulent and non-virulent *Shigella* spp. (both *S. flexneri* and *S. dysenteriae* 1). Virulent strains will be defined as strains inducing keratoconjuctivitis in guinea pigs and this property will be correlated with the ability to bind Congo red. In order to make direct comparison, we will use Congo red positive strains which are virulent with their respective non-virulent Congo red negative derivatives. Antisera prepared against bacterial OMP preparations will be used in crossed immunoelectrophoresis and Western-blot assays in homologous and hetrologous experiments. In addition, we will absorb the anti-virulent OMP sera with non-virulent OMP antigen, thereby making a sera specific for antigens associated with virulence. The absorbed and unabsorbed sera will also be used to determine if OMP antigens are common between *Shigella* and Congo red positive *E. coli* strains. Results from the study will help to define OMP antigens of *Shigella* and their relation to virulence which may assist in the design of a vaccine for shigellosis.

The study does not involve any human subject. Items 1 through 8 are not applicable.